Neurotrophic factor intervention restores auditory function in deafened animals

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A primary cause of deafness is damage of receptor cells in the inner ear. Clinically, it has been demonstrated that effective functionality can be provided by electrical stimulation of the auditory nerve, thus bypassing damaged receptor cells. However, subsequent to sensory cell loss there is a secondary degeneration of the afferent nerve fibers, resulting in reduced effectiveness of such cochlear prostheses. The effects of neurotrophic factors were tested in a guinea pig cochlear prosthesis model. After chemical deafening to mimic the clinical situation, the neurotrophic factors brain-derived neurotrophic factor and an analogue of ciliary neurotrophic factor were infused directly into the cochlea of the inner ear for 26 days by using an osmotic pump system. An electrode introduced into the cochlea was used to elicit auditory responses just as in patients implanted with cochlear prostheses. Intervention with brain-derived neurotrophic factor and the ciliary neurotrophic factor analogue not only increased the survival of auditory spiral ganglion neurons, but significantly enhanced the functional responsiveness of the auditory system as measured by using electrically evoked auditory brainstem responses. This demonstration that neurotrophin intervention enhances threshold sensitivity within the auditory system will have great clinical importance for the treatment of deaf patients with cochlear prostheses. The findings have direct implications for the enhancement of responsiveness in deafferented peripheral nerves.

Hearing impairment is the most frequent disability of people in industrialized countries, affecting more than one in seven individuals. Most hearing loss is caused by destruction of the sensory cells within the cochlea of the inner ear. In mammals, the auditory cells do not regenerate, nor are there currently effective interventions for their repair. Moreover, in the auditory system, as in other afferent systems, degeneration of the auditory nerve occurs secondary to the loss of the inner ear sensory cells (hair cells), thus aggravating the functional impairment. In the severely and profoundly deaf, the cochlear implant (prosthesis) has been shown to provide an effective habilitative intervention. The cochlear prosthesis consists of one or more electrodes inserted into the fluid space of the inner ear. The implant operates by directly electrically stimulating the auditory nerve, bypassing damaged or missing sensory receptor cells. This device now provides significant speech understanding, with a score for everyday sentence understanding of about 80% without lip reading in the majority of patients implanted (so far more than 40,000 worldwide) (1–3). However, the cochlear prosthesis depends on remaining excitable auditory nerve fibers, and their loss severely compromises the effectiveness of the implant and the hearing benefits it provides. Studies show a clear relationship between the total number of viable auditory neurons available for stimulation and the performance of subjects receiving cochlear implants (4, 5).

The secondary degeneration of the afferent nerve fibers is an unavoidable consequence of damage to sensory receptor cells. With the discovery of nerve growth factors by Rita Levi-Montalcini and colleagues in the 1950s and recent increased understanding of the role of neurotrophins as survival factors in the mature nervous system (6), there have been numerous attempts to define ways to reduce this degeneration, particularly in the visual and auditory systems, as well as to reduce neural degeneration at other sites of the central nervous system (CNS). In the auditory system, efforts to reduce nerve degeneration secondary to loss of the sensory cells have an immediate clinical objective of improving the benefits of auditory neural prostheses to the deaf patient. It would be of great clinical importance if the neurotrophins could be shown to effect excitability of the surviving nerve tissue. This effect has not yet been shown *in vivo* in the CNS. This study tests the hypotheses that neurotrophic factor (NTF) treatment both preserves the auditory nerve after severe peripheral damage and, more importantly, enhances functional excitability within the nervous system. Positive findings would be of general significance in the neuroscience field.

Interventions to enhance survival of the auditory nerve and its cell bodies (spiral ganglion cells, SGCs) have been proposed. It has been demonstrated that direct cochlear infusion of neurotrophic factors such as neurotrophin-3, brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor enhances the survival of SGCs after inner hair cell loss (7–9). Moreover, *in vitro* studies indicate that an interaction among factors may synergistically enhance SGC survival (10). Thus, BDNF and ciliary neurotrophic factor (CNTF) were found to be more effective in promoting the survival of neurons in dissociated cell cultures than either factor individually (11); the combination of BDNF and fibroblast growth factor 1 was demonstrated to be more effective than either agent alone in enhancing SGC survival and in inducing regrowth of their peripheral processes (12).

Although NTFs increase SGC survival after inner hair cell loss, it has yet to be demonstrated that enhanced survival is associated with an enhancement of functional capability. An improvement in auditory system responsiveness will be important if such interventions are to be considered for human application, i.e., to improve the benefits of the cochlear prosthesis, or in future designs of sensory neuroprostheses. We have therefore investigated whether the combination of BDNF and the ciliary neurotrophic factor analogue $CNTF_{Ax1}$ will enhance not only SGC survival but also the responsiveness of the auditory system *in vivo* by using an animal model. The influence of chronic treatment of the inner ear with the combination of NTFs on auditory system responsiveness and SGC survival after deafness was investigated.

Abbreviations: AP, artificial perilymph; eABR, electrically evoked auditory brainstem response; NTF, neurotrophic factor; SGC, spiral ganglion cell; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor.

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Methods

Study Design. The effect of NTFs on auditory function was tested in a guinea pig cochlear prosthesis model. To mimic the clinical situation, all animals were chemically deafened, resulting in complete sensory cell loss and, as a consequence, progressive degeneration of the SGCs and their nerve fibers. After the deafening procedure, NTFs were infused directly to the cochlea of the inner ear. A platinum–iridium electrode was introduced into the cochlea and used to elicit auditory responses just as in patients implanted with cochlear prostheses. Auditory responsiveness was assessed by using electrically evoked auditory brainstem responses (eABRs).

Surgery and Cochlear Infusion. Pigmented guinea pigs (250–500 g; $n = 10$) were deeply anaesthetized (xylazine 10 mg/kg, i.m., ketamine 40 mg/kg, i.m.), and the middle ear was exposed by means of a postauricular approach. Inner ear infusion was accomplished by means of an indwelling microcannula–osmotic pump system (Model 2002, Alza) connected to the cochlea by a cannula inserted into the basal turn slightly lateral to the round window, allowing access to the scala tympani (13). Before implantation, the cannula was preloaded with 4 μl of artificial perilymph (AP) and 24 μl of concentrated 10% neomycin solution; the pump was filled with either AP or BDNF + $CNTF_{Ax1}$. Because the output of the pump is calibrated at 0.5 μl/h, AP was infused into the scala tympani for the first 8 h followed by the 10% neomycin infusion for 48 h. The cochlea was then infused for an additional 12 days with the material in the pump reservoir (i.e., BDNF/$CNTF_{Ax1}$, or AP). The osmotic pump was changed on day 15, and the infusion continued until experimental day 29. In five subjects, deafening (i.e., neomycin infusion) was followed by cochlear infusion for 26 days with a combination of BDNF (100 μg/ml) and $CNTF_{Ax1}$ (100 ng/ml). Five animals served as controls and received AP (containing 137 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 11 mM glucose) instead of NTFs for the same period. Otherwise, the protocol was identical.

All animal procedures were performed in accordance with national regulations for care and use of animals (Stockholm Northern Animal Care and Use Committee approval no. N154/98).

Auditory Responses. At the time of pump implantation, a platinum–iridium ball electrode (Pt–Ir 90%/10%, 250-μm diameter) was inserted through the round window membrane and placed ≈1.5 mm into the scala tympani to elicit eABRs. The return wire (125-μm diameter, Pt–Ir) was placed against the occipital bone beneath the dorsal neck muscles. A percutaneous connector was cemented to the dorsal skull with carboxylate cement. The eABRs were recorded in anaesthetized animals in a soundproof room by using the method described by Hall (14), in which responses to alternate polarity current pulses are summed, each pair providing charge balancing. Averages of 2,048 responses, to 50-μs computer-generated monophasic current pulses, presented at 50 pulses per second with an alternating polarity, were recorded between the vertex screw and a reference electrode inserted s.c. just below the contralateral ear. A needle inserted at the right lower extremity served to ground the subject. The eABRs typically demonstrated a classic waveform, consisting of five positive waves. Thresholds could be determined from waves I and III in most recordings. However, as wave I frequently was obscured by the electrical artifact, wave III (P3) was routinely used for determining the eABR threshold. Threshold was defined as the lowest stimulus level, in 50-μA steps, that evoked a replicable waveform (typically >0.4 μV). The eABRs were assessed at weekly intervals throughout the treatment period. The final eABR, followed by the killing of each subject, was performed on day 31.

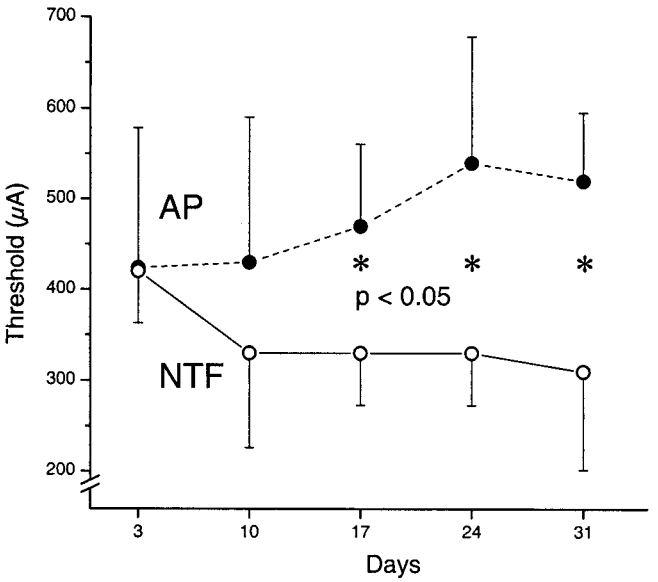

Fig. 1. Mean and SD of the eABR thresholds observed in the AP and NTF treatment groups at different days after the onset of deafness. On day 31, the thresholds for the AP group ranged from ≈450 to 650 μA, whereas the thresholds for the NTF group ranged from ≈150 to 400 μA. There was no overlap between the two treatment groups. The mean eABR thresholds were significantly different between these groups on days 17, 24, and 31 ($P = 0.019$, 0.014, and 0.007, respectively; Student's t test).

Histology. To determine the survival of SGCs, the animals were deeply anaesthetized and fixed by cardiac perfusion (3% glutaraldehyde in phosphate buffer). After decalcification in EDTA and embedding in paraffin, 6-μm sections were cut in a paramodiolar plane. Every fourth section was mounted on a glass slide and stained with toluidine blue. Six sections were randomly selected from the 10 most mid-modiolar sections for each animal and used for quantitative analysis of SGCs. The counting was performed double blind. All neurons meeting size and shape criteria to be considered type I SGCs within each profile of Rosenthal's canal from base to apex of the cochlea were counted. The outline of the Rosenthal's canal profile was then traced to generate a SGC density, expressed as the density of SGC for an area of 10,000 μm^2.

Statistics. Statistical assessment of differences in SGC density and eABR thresholds between the groups was performed by using ANOVA and the Student's t test.

Results

eABRs. Immediately after the deafening procedure, there was little difference between the eABRs evoked in the two groups of subjects. However, substantial changes occurred during the course of the treatment. Fig. 1 illustrates the mean and standard deviation of the threshold of the eABR in μA for each treatment group. In the group treated with AP, eABR thresholds demonstrated a small but systematic increase throughout the treatment period after the deafening procedure and electrode implantation. However, in the deafened subjects treated with BDNF + $CNTF_{Ax1}$, eABR thresholds showed a systematic decrease throughout the treatment period. The rate of change in the threshold of the NTF-treated group was greatest immediately after treatment. Analysis of the slope of threshold change demonstrated an average slope of −27.3 μA/day for the NTF group. A significant decrease in threshold was observed between days 3 and 17 (paired Student's t test, $P = 0.009$) and days 3 and 24 ($P = 0.009$). For the AP group, the largest change (threshold elevation) was observed between 17 and 24 days, which

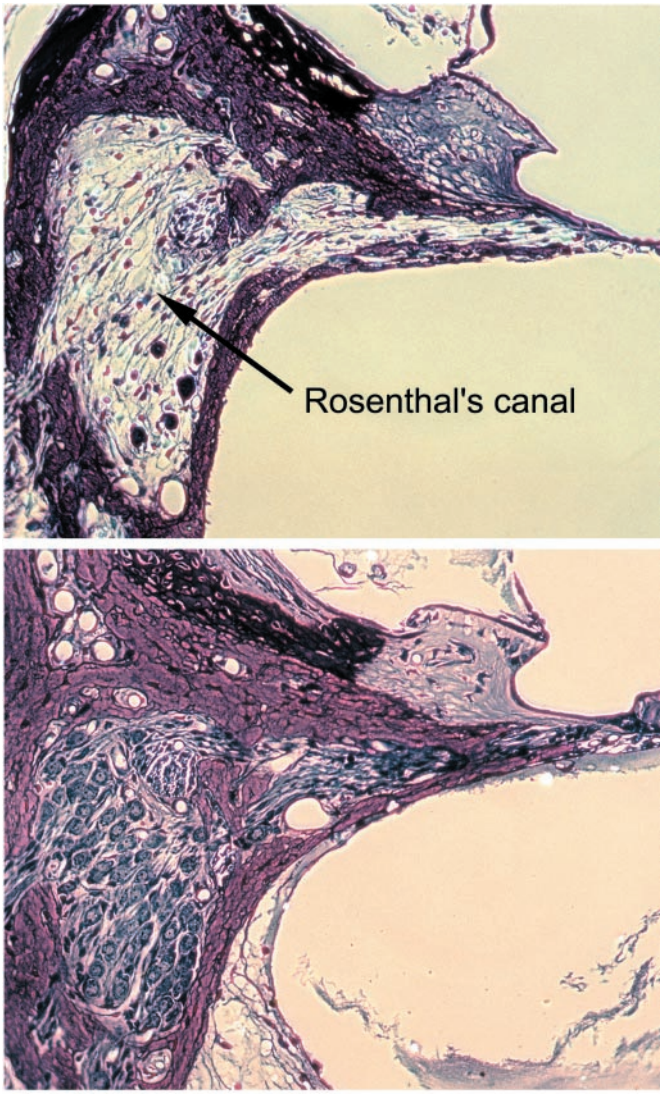

Fig. 2. Representative sections of Rosenthal's canal in the base of the cochlea from an AP-treated subject (*Upper*) and a subject of the NTF-treated subject (*Lower*). There is a clear difference in the survival of SGCs in these two subjects.

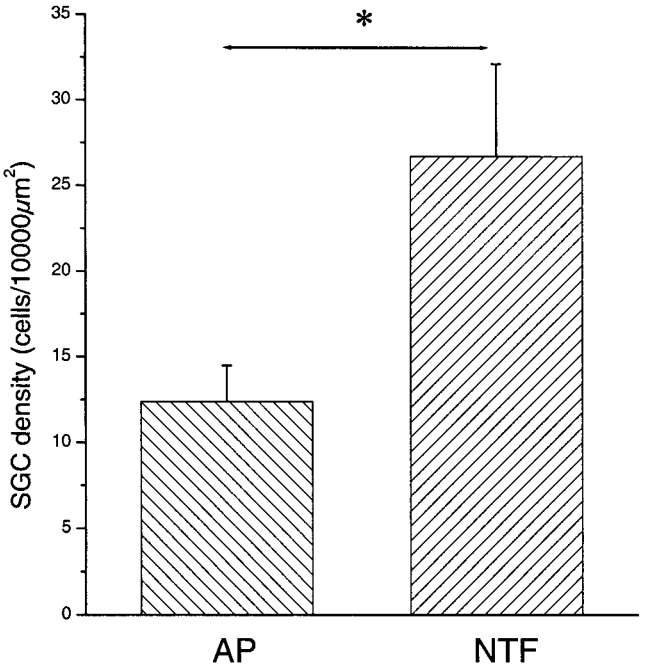

Fig. 3. The observed mean and SEM of SGC density in AP- and NTF-treated groups. In the AP group, SGC density ranged from ≈5,000 to 20,000 cells per 10,000 μm^2. In the NTF-treated group, survival ranged from 11,000 to 39,000 cells per 10,000 μm^2. The mean difference was significant. *, $P < 0.05$.

is consistent with the occurrence of significant SGC degeneration (15). There were significant differences in mean eABR thresholds between the two treatment groups on measurement days 17, 24, and 31 ($P < 0.05$).

SGC Survival. Intracochlear treatment with the combination of BDNF and $CNTF_{Ax1}$ resulted in an enhanced survival of SGCs in the treated cochleae compared with cochleae receiving AP. This difference is clearly seen in Fig. 2, showing the spiral ganglion regions in an AP-treated cochlea and in a cochlea that was infused with NTFs. Across the whole cochlea, for the group receiving AP, the mean density of SGCs was 12.4 (SEM = 2.1) whereas for the group receiving BDNF and $CNTF_{Ax1}$, the mean density (26.7, SEM = 5.4) was significantly higher (at $P < 0.05$; Fig. 3). There was a relationship between the recorded eABR thresholds and measured SGC density. The subjects with the greater density of surviving SGC also demonstrated the lower eABR thresholds.

Discussion

In human patients, cochlear prostheses are usually implanted more than 6 months after deafness. This period of deafferentation will, at least in animal models, lead to a significant loss of auditory nerve cells (15, 16). In humans, however, the process is suggested to be slower (17). Because the benefits of a cochlear prosthesis have been shown to be strongly correlated to auditory nerve survival, it is important to implant the profoundly deaf candidate for a prosthesis as soon as possible after deafness.

Several electrophysiological studies have demonstrated a relationship between the number of SGCs and amplitude growth function of the eABR (14, 15, 18). Although Hall (14) reported that the amplitude of the first positive wave was correlated with the number of SGCs, these previous studies found no relation between SGC survival and eABR threshold. In this study, significant differences were observed.

The results show increased sensitivity associated with an increased SGC density in NTF-treated animals. We have demonstrated, in the central nervous system, that a neurotrophin intervention may result in enhanced sensitivity of deafferented neural tissue *in vivo*. The increase in the mean SGC density in the NTF-treated group compared with the AP-treated group is consistent with the observed difference in eABR thresholds. Because the auditory brainstem responses can be assessed noninvasively in humans, these results indicate that eABR threshold measurements may provide a quantitative assay of SGC density in the cochlear implant candidate. There is, however, an alternative explanation if one considers the magnitude of the eABR as a measure of synchronized activity across the SGCs responding to the electrical stimulus. It is possible that one may obtain a small-magnitude eABR if the number of SGCs were the same but the neurons did not respond in a synchronized way. Thus, the eABR may be indicative of not only the number of responding SGCs, but also the extent of synchronization of their responses. This study, however, can not resolve this issue.

The present study supports other recent investigations showing that neurotrophins enhance SGC survival after deafferentation *in vivo* (7–9). More importantly, this investigation demonstrated that the prevention of loss of SGCs by NTF significantly improves

hearing sensitivity to electrical stimulation. Clearly there are many practical hurdles to the safe application of NTF in the inner ear of humans. However, when safety issues are resolved, this study supports the clinical utility of NTF as adjunct treatment at the time of cochlear prosthetic surgery.

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1. Skinner, M. W., Clark, G. M., Whitford, L. A., Seligman, P. M., Staller, S. J., Shipp, D. B., Shallop, J. K., Evringham, C., Menapace, C. M., Arndt, P. L., *et al.* (1994) *Am. J. Otol.* **15** Suppl. 2, 15–27.
2. Kiefer, .J., Muller, J., Pfenningdorff, T., Schön, F., Helms, J., von Illberg, C., Baumgartner, W., Gstöttner, W., Ehrenberger, J., Arnold, W., *et al.* (1996) *ORL Otorhinolaryngol. Relat. Spec.* **58,** 127–135.
3. Illg, A., von der Haar-Heise, S., Goldring, J. E., Lesinski-Schiedat, A., Battmer, R. D. & Lenarz, T. (1999) *Ann. Otol. Rhinol. Laryngol.*, Suppl. **177,** 93–98.
4. Nadol, J. J., Young, Y. S. & Glynn, R. J. (1989) *Ann. Otol. Rhinol. Laryngol.* **98,** 411–416.
5. Gantz, B. J., Woodworth, G. G., Knutson, J. F., Abbas, P. J. & Tyler, R. S. (1993) *Ann. Otol. Rhinol. Laryngol.* **102,** 909–916.
6. Mattson, M. P. & Furukawa, K. (1998) *Perspect. Dev. Neurobiol.* **5,** 337–352.
7. Staecker, H., Kopke, R., Malgrange, B., Lefebvre, P. & Van De Water, T. R. (1996) *NeuroReport* **7,** 889–894.
8. Ernfors, P., Duan, M. L., ElShamy, W. M. & Canlon, B. (1996) *Nat. Med.* **2,** 463–467.
9. Miller, J. M., Chi, D. H., O'Keeffe, L. J., Kruszka, P., Raphael, Y. & Altschuler, R. A. (1997) *Int. J. Dev. Neurosci.* **15,** 631–643.
10. Hegarty, J. L., Kay, A. R. & Green, S. H. (1997) *J. Neurosci.* **17,** 1959–1970.
11. Hartnick, C. J., Staecker, H., Malgrange, B., Lefebvre, P. P., Liu, W., Moonen, G. & Van De Water, T. R. (1996) *J. Neurobiol.* **30,** 246–254.
12. Altschuler, R. A., Cho, Y., Ylikoski, J., Pirvola, U., Magal, E. & Miller, J. M. (1999) *Ann. N.Y. Acad. Sci.* **884,** 305–311.
13. Brown, J. N., Miller, J. M., Altschuler, R. A. & Nuttall, A. L. (1993) *Hear. Res.* **70,** 167–172.
14. Hall, R. D. (1990) *Hear. Res.* **49,** 155–168.
15. Jyung, R. W., Miller, J. M. & Cannon, S. C. (1989) *Otolaryngol. Head Neck Surg.* **101,** 670–682.
16. Leake, P. A. & Hradek, G. T. (1988) *Hear. Res.* **33,** 11–33.
17. Otte, J., Schuknecht, H. F. & Kerr, A. G. (1978) *Laryngoscope* **88,** 1231–1246.
18. Smith, L. & Simmons, F. B. (1983) *Ann. Otol. Rhinol. Laryngol.* **92,** 19–23.